Simultaneous Detection of CYP3A4, CYP2D6 and CYP2C9 Metabolites with a Single, Sensitive, LC/MS/MS Method

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Introduction

Cytochrome P450s (CYPs) are heme-containing enzymes responsible for initial metabolism of xenobiotics within the human body[1]. CYPs are part of the Phase I (oxidative) metabolism within the body, which introduces a hydrophilic site on the metabolized drug enabling either easy elimination of the drug or further conjugation by Phase II enzymes, such as UDPGTs, sulfotransferases or amino acid-conjugation (among others)[2]. Recently there has been increased interest in understanding CYPs to discover the underlying mechanisms of drug-drug interactions[3], drug side effect(s)[4] and toxicity (including hepatotoxicity). Also relevant to CYP testing is the discovery that polymorphisms in the genetic sequences expressing these enzymes leads to populations of people whose ability to metabolize certain classes of drugs may be attenuated in comparison to other races/populations[5].

In this application note LC-ESI-MS/MS is used to simultaneously characterize three drug metabolites for the detection of three different cytochrome P450's enzymatic activities; the hydroxylation of midazolam (CYP3A4), hydroxylation of tolbutamide (CYP2C9) and O-demethylation of dextromethorphan (CYP2D6), see Figure 1. This assay could be used to analyze CYP3A4, CYP2D6 and CYP2C9 activity in vitro for kinetic studies (e.g., liver microsome inhibition studies) or possibly for detection of these metabolites in more complex matrices (plasma or urine) after SPE.

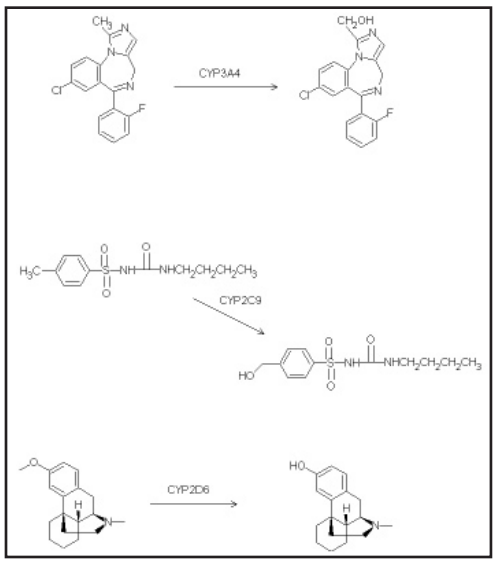

Figure 1. Cytochrome P450 catalyzed reactions

Instrumentation

- Varian ProStar 210 Solvent Delivery Modules (2)
- Varian 1200L LC/MS equipped with an ESI source
- Varian ProStar 430 AutoSampler

Materials and Reagents

All chemicals were reagent or HPLC grade from Sigma-Aldrich Corporation (St. Louis, MO) with the exception of midazolam and α-hydroxymidazolam standard solutions (1 mg/mL and 100 µg/mL in methanol, respectively) from Cerilliant (Round Rock, TX).

Sample Preparation

Stock solutions were prepared in HPLC grade water (dextrorphan) or methanol (4-hydroxytolbutamide) at 1 mg/mL. All diluted samples were prepared in 50:50 methanol: water (HPLC grade).

Mass Spectrometry Conditions

Ionization Mode	ESI positive
Collision Gas	2.0 mTorr Argon
API Drying Gas	25 psi at 350 °C
API Nebulizing Gas	51 psi
Scan Time	1 sec
SIM Width	0.7 amu
Needle	5000V
Capillary	44V
Shield	600V
Detector	1800V

LC Conditions

Column	Varian MonoChrom MS 5 µm 50 x 2 mm (Varian Part #A2080050X020)			
Solvent A	0.1% Formic acid in Deionized Water			
Solvent B	Acetonitrile			
LC Program	Time (min:sec)	%A	%B	Flow (mL/min)
	0:00	90	10	0.25
	2:30	60	40	0.25
	3:00	60	40	0.25
	5:30	10	90	0.25
	6:00	10	90	0.25
	6:30	90	10	0.25
	7:00	90	10	0.25

Injection Volume: 10 µL

MS/MS Scan Parameters

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)
Dextrorphan	258.6	157	-32
4-hydroxy-tolbutamide	287	89	-26
α-hydroxy-midazolam	342.2	168	-36

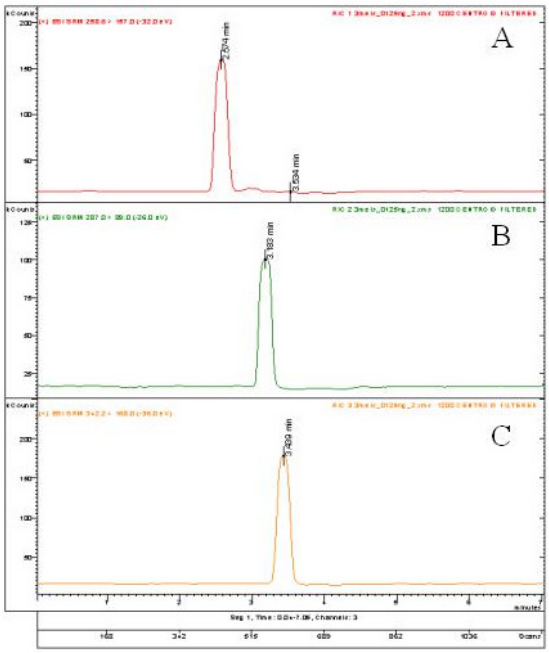

Figure 2. Chromatograms of A) Dextrorphan, B) 4-Hydroxytolbutamide, C) 1'-Hydroxymidazolam (1 ng each)

Discussion

Testing of drug metabolizing enzymes (such as cytochrome P450s) is becoming increasingly important for the evaluation of new chemical entities (NCEs) before evaluation of the new drug in the clinic. Newly synthesized compounds that display proper biological effects on the target of choice in vitro, can and should be evaluated for their effects on drug metabolizing enzymes before an expensive scale-up of the new drug takes place for clinical testing. CYP3A4, 2D6 and 2C9 were chosen for our evaluation because 45-60% of the drugs currently on today's market are metabolized by CYP3A isoforms alone[6], and CYP2D6 and CYP2C family members are subject to polymorphisms leading to populations of people that are poor metabolizers[7]. Having a combined method for all three would, therefore, be useful for testing samples from all of these enzymes at one time, eliminating the need to switch solvents and/or whole methods to evaluate these enzymes.

Good separation of the metabolites is obtained, in less than 5 minutes, on a Varian MonoChrom MS column with 0.1% formic acid (Solvent A) and acetonitrile (Solvent B) with the gradient mentioned (see LC Method, above). Figure 2A – C, shows the individual peaks as detected by single reaction mode (SRM) in the 1200L. Limit of detection (LOD) for each of these metabolites was determined to be: 0.5, 0.25 and 0.25 pg (4-hydroxytolbutamide, -hydroxymidazolam and dextrorphan, respectively) and the limit of quantitation for each of these metabolites was determined to be: 1.0, 0.5 and 0.5 pg, respectively.

Calibration curves for the three metabolites were run in triplicate over the range of 1000 to 1 pg and are shown in Figures 3 - 5. The original xenobiotics can also be quantified, if desired, to determine percent usage of the drug (data not shown). Excellent linearity is seen within this range with the average correlation coefficient being 0.993. Accuracy in retention times of the metabolites is also excellent with an average 1.15% RSD, the dextrorphan being slightly more variable (2.74% RSD).

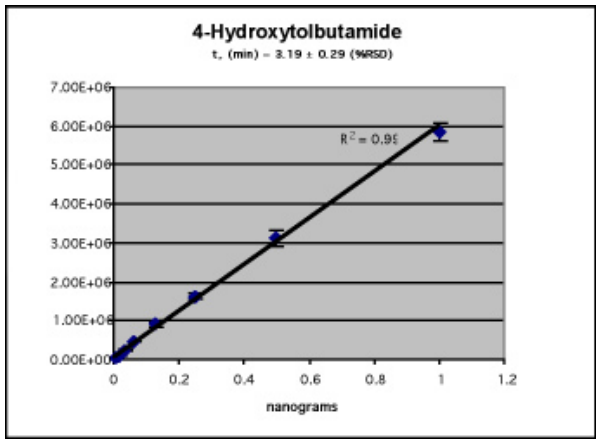

Figure 3. 4-Hydroxytolbutamide Calibration Curve (1 – 1000 pg)

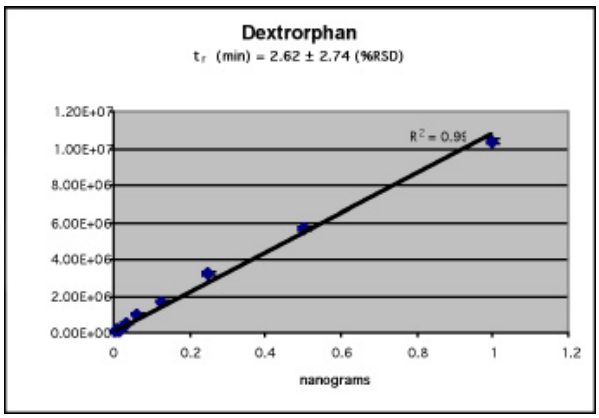

Figure 4. Dextrorphan Calibration Curve (1 – 1000 pg)

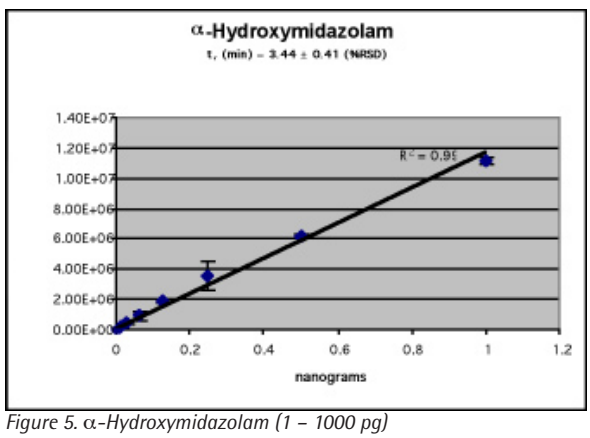

Figure 5. α-Hydroxymidazolam (1 – 1000 pg)

Conclusion

With this application note we have demonstrated a single method for the separation and quantification of the enzymatic activities of the three most important Cytochrome P450 enzymes (3A4, 2D6 and 2C9). This method can be used for pre-clinical evaluation of NCEs as inhibitors of CYPs or quantification of these metabolites from clinical samples after SPE.

References

1. Wrighton, S. A., Stevens, J. C. Crit. Rev. Toxicol. 1, 1-21 (1992).
2. Kwak, M. K. et al. Mutat. Res. 480-481, 305-15 (2001).
3. Honig, P. K. et al. J. Am. Med. Assoc. 269, 1513-1518 (1993).
4. Kuntzman, R., et al. J. Pharmacol. Exp. Ther. 152, 151 (1996).
5. Lee, C.R., Goldstein, J.A., Pieper, J.A. Pharmacogenetics 3, 251-63 (2002).
6. Wojnowski, L. Ther. Drug. Monit. 26, 192-9 (2004).
7. Brockmoller J., et al. Pharmacogenomics 1, 125-151 (2000).

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